

Podocytes that detach in experimental membranous nephropathy are viable¹

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Podocytes that detach in experimental membranous nephropathy are viable.

Background. Podocyte loss contributes to the development of glomerulosclerosis. Although podocytes have been detected in the urine in certain glomerular diseases, the viability of detached cells is not known.

Methods. Urine was collected from rats with experimental membranous nephropathy [passive Heymann nephritis (PHN) model], centrifuged, and following resuspension in tissue culture media, cells were seeded onto collagen-coated tissue culture plates. Cells were grown under typical cell culture conditions. Cell number was measured, the cell type was identified by immunostaining with specific antibodies, and cell morphology was assessed by light and electron microscopy.

Results. Cells obtained in the urine from PHN rats were positive for synaptopodin, nephrin, podocin, WT-1, and GLEPP1 (podocyte-specific antigens). When grown ex vivo under cell culture conditions, cells obtained in the urine from PHN rats adhered to tissue culture plates, and expressed podocyte-specific proteins at the mRNA [reverse transcription-polymerase chain reaction (RT-PCR)] and protein (immunostaining) level. Cells did not stain with antibodies to mesangial (OX-7), tubular (Tamm-Horsfall protein) and endothelial (RECA) cells. Electron microscopy showed the presence of foot processes, and podocytes from PHN rats stained positive for C5b-9. Although podocyte number increased transiently during the first 5 days ex vivo, apoptosis increased significantly thereafter, reducing overall cell number.

Conclusion. Rats with experimental membranous nephropathy shed podocytes into the urine that attach to tissue culture plates ex-vivo, and proliferate. These results suggest that detached podocytes are viable. These results add new perspectives into our understanding of podocyte loss in the development of glomerulosclerosis.

Podocytes, also called visceral glomerular epithelial cells, are terminally differentiated and highly specialized

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cells lining the outer aspect of the glomerular basement membrane [1, 2]. It has been well established that a decrease in podocyte number contributes to the development of glomerulosclerosis in most forms of nephrotic syndrome. However, it is not clear why podocyte number decreases in immune-mediated disease such as membranous nephropathy, or in nonimmune diseases such as diabetic nephropathy [3–5]. One explanation is that podocytes are unable to proliferate and replace those lost, because they are terminally differentiated [6, 7]. Another possibility is podocyte apoptosis, which has been shown in experimental and human glomerular diseases [8–11]. A third plausible mechanism underlying podocyte loss is detachment of cells from the glomerular basement membrane. Indeed, studies have shown that podocytes are present in the urine of patients with a variety of glomerular diseases [12–18].

However, it is unclear if podocytes in the urine are viable and biologically active, or if detached podocytes are nonviable. Accordingly, the goal of this study was to determine the viability of podocytes that detach in experimental membranous nephropathy. Our data show that podocytes present in the urine are viable, have the ability to attach to tissue culture plates ex vivo, and have a limited proliferative capacity.

METHODS

Passive Heymann nephritis (PHN) model

To determine if viable podocytes detach from the glomerular basement membrane following complement (C5b-9)-mediated injury, the passive Heymann nephritis (PHN) model of experimental membranous nephropathy was induced in male Sprague-Dawley rats (Simson; Gilroy, CA, USA) weighing 180 to 200 g by intraperitoneal injection of sheep antibody to Fx1A (5 mL/kg body weight) as previously described [7]. Control animals were injected with normal sheep serum. A 24-hour urine collection was performed on control and PHN rats prior to sacrifice, and urine protein excretion was determined by

the sulphosalicylic method as previously reported [19]. Urine collected by this method was used to isolate cells in the urine (see below). Urine was also obtained by bladder puncture (see below) from PHN and control animals on days 1, 3, 5, 6, 10, and 20 ($N = 6$ per group/time point). At sacrifice, renal biopsies were taken from control and PHN rats.

Urine collection and cell culture

In order to collect urine from individual control and diseased rats by bladder puncture, animals were anesthetized by ether inhalation and an intramuscular injection of a mixture containing ketamine (50%), xylazine (25%), and acepromazine (15%) in Ringer's solution (10%). Following anesthesia, a butterfly needle was inserted into the tail vein, and a bolus of 6 to 8 mL of normal saline was injected intravenously, over 2 to 5 minutes, to induce a forced diuresis leading to immediate and predictable bladder filling. Our pilot studies showed that cells were also detected in urine collected for 12 hours and 24 hours in animals that did not receive saline. However, using timed collections, urine volumes were significantly smaller, and at times, undetectable. Moreover, collecting urine for 12 hours and 24 hours was also hampered by the co-collection of feces and dander. Accordingly, to ensure that we could predictably collect adequate urine that was not contaminated, all control and diseased animals in this study received saline boluses at each time point studied.

Following the administration of intravenous fluid, the abdomen was shaved and opened through a longitudinal incision. The urinary bladder was localized, and a sterile insulin syringe was used to puncture the bladder. Urine (0.8 to 1 mL) was removed by syringe, and rapidly transferred to a sterile 12 mL tube. The urine acidity was partially neutralized by adding 11 mL of prewarmed Hanks' solution. Following centrifugation at 1200 rpm at room temperature for 5 minutes, the supernatant was removed by careful suction and discarded, and the cell pellet resuspended in 12 mL of cell culture Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Summit Biotechnology, Ft. Collins, CO, USA), penicillin (100 U/mL), streptomycin (100 U/mL), and glutamine (2 mmol/L) (Irvine Scientific, Santa Ana, CA, USA). This medium is used to culture rat podocytes *in vitro*. The resuspended pellet was divided into 2 mL aliquots and seeded in individual wells in a six-well tissue culture plate (Nunc; Denmark) coated with collagen I (BD Biosciences; Two Oak Park, MA, USA). Tissue culture plates were incubated at 37°C in 95% air/5% CO₂ overnight. The following day, red blood cells and nonattached cells were removed by washing with Hanks' solution. Adherent cells were subsequently grown at 37°C in DMEM medium containing 5% FBS, which was replaced every 3 days.

Documentation of cell attachment

After 24 hours of cell culture, cells that formed "colonies" (defined as >five adherent cells that were making cell-cell contact) were marked by circling the base of the tissue culture plate. Twice-daily inverted microscopy was performed to count the number of adherent cells within each colony and to document morphologic changes. Cells with typical apoptotic morphologic features were also counted, as we have reported previously [20].

Immunostaining

In vivo. In PHN rats, detection of sheep immunoglobulin G (IgG) and C5b-9 in glomeruli was performed by direct (sheep IgG) and indirect (C5b-9) immunofluorescent staining on 4 µm frozen renal biopsy sections fixed in ether/alcohol as described elsewhere [7]. Sections were stained with fluorescein conjugated rabbit antisheep IgG (Organon Teknica Corporation, West Chester, PA, USA) or biotinylated 2A1, a murine monoclonal antibody to rat C5b-9, followed by fluorescein-conjugated streptavidin (Amersham, Arlington Heights, IL, USA). Tissue sections fixed in methyl carnoys solution were also used for periodic acid-Schiff (PAS) staining.

Immunofluorescent staining of cultured cells. The cell type growing *ex vivo* on tissue culture plates from control and membranous rats was determined by immunofluorescent staining at day 1 and day 8 after seeding. Adherent cells were fixed at -20°C in methanol:acetone (1:1) for 20 minutes, air dried for 15 minutes, rehydrated with phosphate-buffered saline (PBS), and permeabilized with 0.1% NP40. Cells were incubated overnight at 4°C with the following podocyte-specific antibodies: (1) WT-1 (mouse monoclonal; clone sc-7385) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100 dilution); (2) synaptopodin (mouse monoclonal; clone G1) (gift of Dr. Peter Mundel, Albert Einstein, Bronx, NY) (1:20 dilution); (3) nephrin (rabbit polyclonal) (gift of Dr. Harry Holthöfer, University of Helsinki, Helsinki, Finland) (1:100 dilution); (4) GLEPP1 (rabbit polyclonal) (gift of Dr. Roger Wiggins, University of Michigan, Ann Arbor, MI) (1:5 dilution); and (5) podocin (rabbit polyclonal) (gift of Dr. Peter Mundel, Albert Einstein, Bronx, NY) (1:50 dilution).

To determine if cells other than podocytes were growing *ex vivo*, immunostaining was also performed with antibodies to (1) Thy1.1, a mesangial cell specific antigen (clone MRC OX-7) (Serotec, Ltd., Oxford, England); (2) RECA-1, an endothelial cell-specific antibody (clone HIS52) (Serotec Ltd.); (3) Tamm-Horsfall protein, specific for thick ascending limb (gift of Dr. J. Hoyer, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA) [21]; and (4) a fluorescein isothiocyanate (FITC)-labeled lectin from *Tetragonolobus purpureas*, specific for proximal tubular cells (Sigma Chem-

ical Co., St. Louis, MO, USA) [22]. We tested the specificity of all the antibodies on frozen kidney sections by immunofluorescence as described above for cultured cells.

A secondary biotinylated horse antimouse (clone BA-1000) (Vector Laboratories, Burlingame, CA, USA) (1:100 dilution) or goat anti-rabbit (clone BA-2001) (Vector Laboratories) (1:200 dilution) antibody was used, followed by a fluorescein-conjugated streptavidin antibody (Amersham).

To determine the presence of C5b-9 on detached cells, and to determine if podocytes were present in the urine obtained by cytospin prior to growing *ex vivo*, urinary cells from control and PHN rats (day 5) were fixed for 20 minutes in methanol:acetone (1:1) at -20°C , air dried for 15 minutes, rehydrated with PBS, and stained with a biotinylated primary antibody directed against C5b-9 over night (1:50 dilution), which had been successfully used in the staining of frozen tissue from PHN day 5 rats in the past. A secondary staining with streptavidin-fluorescein (RPN 1232) (Amersham) (1:100 dilution) for 30 minutes at room temperature was performed. These cells were also immunostained with antibodies directed against WT-1, GLEPP1, and nephrin.

Finally, to determine if podocyte number decreases in PHN rats, WT-1 indirect immunoperoxidase immunostaining was performed on biopsies from control and PHN rats as previously reported [23]. The number of WT-1 staining cells (i.e., podocytes) in 20 to 50 glomeruli were counted in individual control and PHN rats ($N = 6/\text{time point/group}$) at 1, 2, 3, 4, and 6 months. In order to correlate podocyte number with proteinuria, regression analysis was performed. Regression analysis was also performed to correlate the number of podocytes in the urine and proteinuria on day 5.

Electron microscopy. Scanning electron microscopy was also performed on adherent cells fixed in half-strength Karnovsky's solution after 8 days in cell culture.

Methods for reverse transcription-polymerase chain reaction (RT-PCR)

In order to ensure that the immunostaining results were confirmed at the mRNA level, we performed RT-PCR on RNA obtained from cells in the urine of control and PHN rats and on RNA obtained from adherent cells grown under cell culture conditions. Total RNA was isolated from cells immediately after collection from the urine of individual rats, and on day 2 growing in culture *ex vivo*, using Tri Reagent (Sigma Chemical Co.) according to the manufacturer's instructions. RQ1 RNase-free DNase (Promega, Madison, WI, USA) was used to remove genomic DNA. cDNA synthesis was carried out with SuperScript First-Strand Synthesis System (Life Technologies, Gibco BRL, Rockville, MD, USA) according to the manufacturer's instructions. Negative control PCR reactions used samples that did not have reverse transcriptase added to the first strand synthesis

reaction or H_2O added instead of cDNA. Alternatively, RNA samples were treated with RNase-free DNase before the first-strand synthesis. Total RNA from normal whole rat kidney was used as a positive control.

PCR for WT-1 was performed with sense primer 5'-gtcctccccgccagccag-3' and antisense primer 5'-tgtagggcggtccctcaggagc-3'. PCR conditions were a MgCl_2 concentration of 2.25 mmol/L, 35 to 40 cycles with 45 seconds at 95°C , 45 seconds at 66°C , 1 minute at 72°C , followed by 10 minutes at 72°C . Nephrin sense primer was 5'-gttcagctggagagactgg-3' and antisense primer 5'-aatcgagcagacaagacgaac-3'. Podocin sense primer was 5'-tctccaaacctgttgacc-3' and antisense primer was 5'-gcgtgtgtccatctgtgac-3'. For nephrin and podocin, PCR conditions were 2 mmol/L MgCl_2 , 35 to 40 cycles with 45 seconds at 94°C , 45 seconds at 55°C , 1 minute at 72°C , followed by 10 minutes at 72°C .

For PCR of synaptopodin sense primer was 5'-gcagaggagtgagggtccag-3' and antisense primer was 5'-gatgccac taggtgtcagg-3' with 2 mmol/L MgCl_2 and for PCR of GLEPP1 sense primer was 5'-atggttcagtcggctctttg-3' and antisense primer was 5'-tcaccgagtcctctgtgtg-3' with 1.5 mmol/L MgCl_2 were used. Synaptopodin and GLEPP1 PCR conditions were 40 cycles with 45 seconds at 94°C , 45 seconds at 57°C , 1 minute at 72°C , followed by 10 minutes at 72°C . All PCR reactions used Taq DNA Polymerase (Promega).

RESULTS

Cells isolated from the urine of PHN rats are podocytes

As expected, urinary protein increased in PHN rats, but not in control rats injected with normal sheep serum (Fig. 1A). To test the hypothesis that podocytes detach from the glomerular basement membrane in PHN rats, immunostaining and RT-PCR was performed for podocyte-specific antigens on cells obtained in the urine from control and PHN rats. One of the anticipated problems with the standard 12-hour or 24-hour urine collection is that if any viable cells were present, they might die at room temperature or if left unattended. Also, the urine collected over 24 hours is often contaminated by feces and dander, and cells are exposed to a low pH. Accordingly, to reliably obtain urine from rats, and to ensure that viability was not compromised, control and diseased animals were injected with a volume of saline intravenously that did not cause any cardiovascular compromise or fluid overload. To exclude an increase in Starling forces underlying the detachment of podocytes from the glomerular basement membrane, these experiments were repeated at least three times, and control rats (injected with normal sheep serum) and healthy normal rats were also injected with saline. Please note that the results described below were identical when obtained using the "standard" 12-hour or 24-hour urine collection. Also

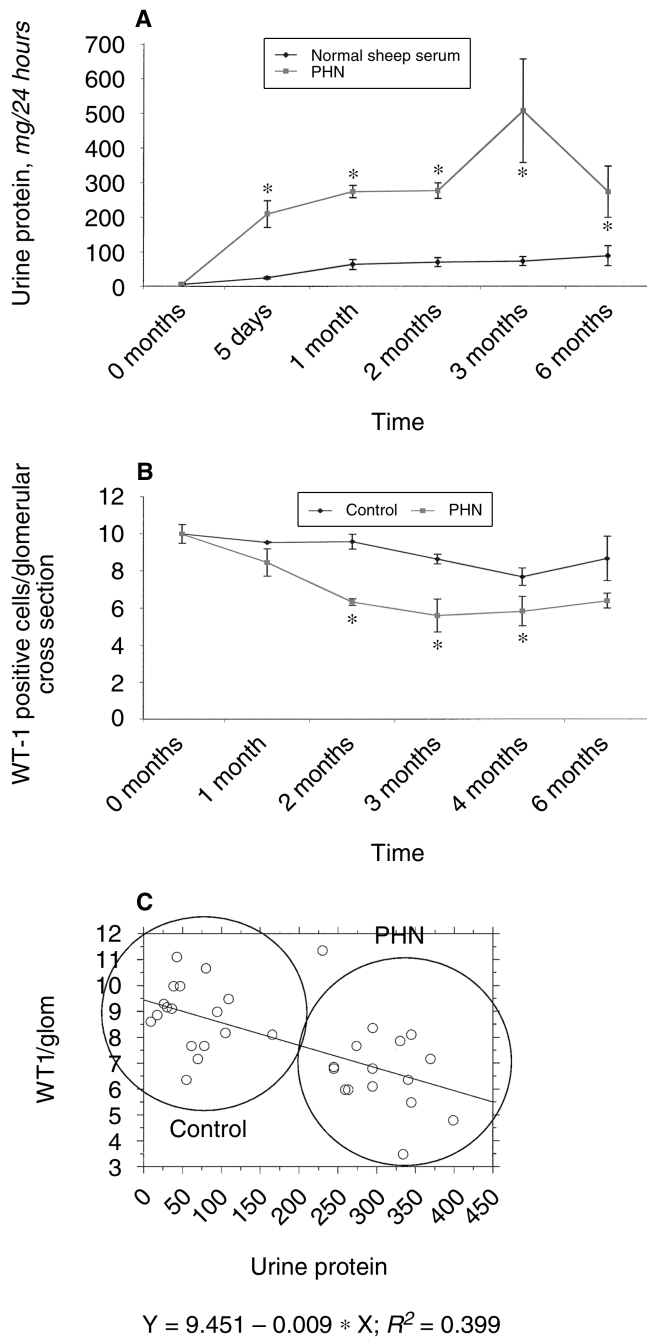


Fig. 1. Proteinuria and cell number in passive Heymann nephritis (PHN). (A) There was a sustained increase in 24-hour urinary protein excretion in PHN rats at each time point studied compared to control rats injected with normal sheep serum. * $P < 0.001$ vs. control (values represent mean \pm standard errors). (B) Podocyte number was measured by counting the number of WT-1-positive cells. Podocyte number decreased in PHN rats, but not in controls. * $P < 0.001$ vs. control. (C) There was a significant correlation between proteinuria and the decrease in podocyte number in PHN rats.

note that sacrificed PHN rats who did not receive saline had viable cells in the urine. In contrast, no cells were present in healthy controls using the same method.

Figure 2 shows that when the urine obtained from

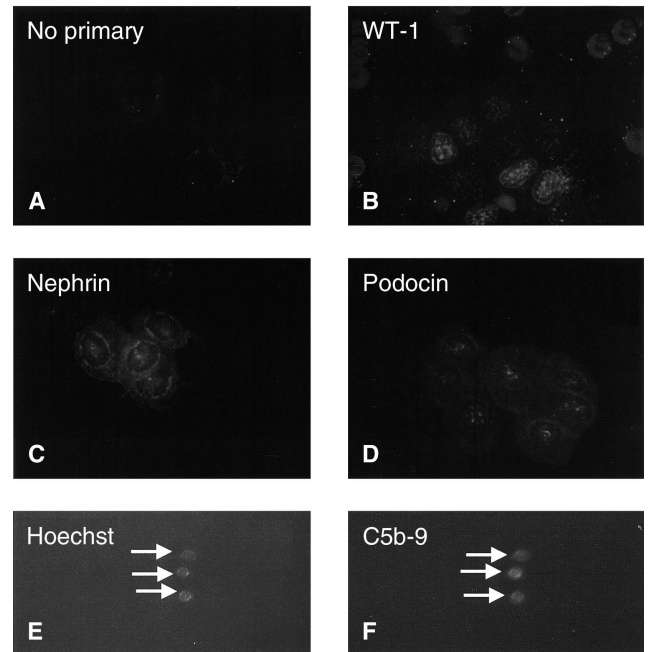


Fig. 2. Immunostaining for podocyte antigens in the urine of passive Heymann nephritis (PHN) rats. Immunostaining was not detected when the primary antibody was omitted, which was used as a negative control (A). Immunostaining for WT-1 (B), nephrin (C), and GLEPP1 (D), podocyte-specific antigens, was detected in cells obtained from the urine of PHN rats, but not from controls (not shown). Double immunostaining for Hoechst and C5b-9 (E) and (F), respectively, in cells in the urine from PHN rats show that detached cells stain positive for C5b-9.

PHN rats was centrifuged, the collected cells stained positive for WT-1, nephrin, and podocin. GLEPP1 staining was also detected (results not shown). Immunostaining for these podocyte-specific antigens was not detected in urine from control rats. Immunostaining for synaptopodin was not detected in PHN rats (data not shown). This result was not a false negative because we showed by immunostaining that these antigens were detected in glomeruli of control and PHN rats (data not shown).

Because of the low protein yield from cells in the urine, the mRNA levels for podocyte-specific antigens were measured by RT-PCR instead of Western blot analysis. Figure 3 shows that mRNA expression for WT-1, GLEPP1, nephrin, podocin, and synaptopodin was detected in cells obtained from the urine of PHN rats. A housekeeping mRNA was not performed because these results were not quantitative, but rather were performed to determine the presence of transcripts. Taken together, these results show that podocytes were detected in the urine from PHN rats (results summarized in Table 1).

Podocyte number decreases in PHN rats

We next asked if the presence of podocytes in the urine leads to a decrease in podocyte number in PHN

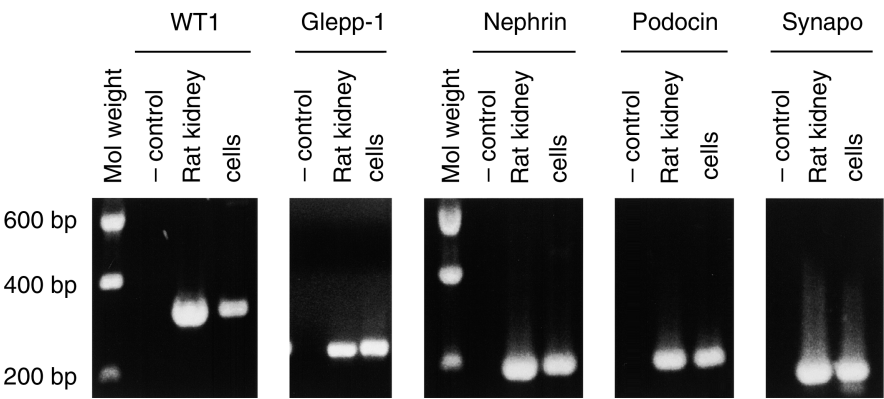


Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) for podocyte antigens in the urine of passive Heymann nephritis (PHN). Total RNA was extracted from the cells obtained in the urine of PHN rats, and subjected to RT-PCR. mRNA expression for WT-1, GLEPP1, nephryn, podocin and synaptopodin (synapo) was detected. RNA from rat kidneys served as positive controls, and replacing the RT mix with H₂O served as negative control.

Table 1. Summary of the expression of podocyte-specific proteins in cells obtained from the urine of passive Heymann nephritis (PHN) rats

	WT-1	Nephryn	Podocin	GLEPP1	Synaptopodin
Staining of nonadherent urinary cells	+	+	+	+	–
RT-PCR of nonadherent urinary cells	+	+	+	+	+
Staining of adherent cells	–	+	+	+	+
RT-PCR of adherent cells	+	–	+	+	–

RT-PCR is reverse transcription-polymerase chain reaction. +, expression detected; –, expression not detected.

rats. Accordingly, we counted the number of cells staining positive for WT-1 in control and PHN rats at different time points. Figure 1B shows that podocyte number decreased progressively in PHN rats, and this reached statistical significance by 2 months of disease ($P < 0.001$ vs. control; $P < 0.001$ vs. normal rat). Podocyte number did not decrease in control rats injected with normal sheep serum.

In order to determine any correlation between podocyte number and proteinuria, regression analysis was performed, and the data are shown in Figure 1C. There was a statistically significant correlation between WT-1–positive cells per glomerulus (i.e., podocytes) and urine protein in PHN rats ($R^2 = 0.986$). Taken together, podocyte number decreases in PHN rats, and this correlates with an increase in proteinuria.

Podocytes in the urine of PHN rats are viable

In order to determine if podocytes detected in the urine of PHN rats were viable, urine collected from control and PHN rats was centrifuged, resuspended in tissue culture media, plated out onto tissue culture plates, and maintained under standard tissue culture conditions. No cells were detected growing in cell culture plates obtained from normal rats, or from control rats injected with normal sheep serum. In contrast, Figure 4 shows that when cells were obtained from the urine of PHN rats and grown in culture, cells were easily identified that were adherent to tissue culture plates within 4 hours of plating. Adherent cells were either single, in clusters

of <five cells, or in “colonies” comprising more than ten cells. Figure 4 shows typical “colonies” of cells in culture after 24 hours of growth. When followed serially over time, it was observed that single cells had moved from their original location to join a “colony” of cells, suggesting that they were motile. Motility can also be seen in Figure 4A and B with cells spreading out centrifugally in all directions from the colony center to the periphery. Figure 4C and D also shows that the number of individual cells within a “colony” increased. Individual cells also increased in size over time. Taken together, viable cells (adherent, motile, and increasing in number) were present in urine from PHN rats, and not in controls.

In order to determine if viable cells were detected in the urine at different times following the induction of PHN, and to determine the peak of this event, urine was collected at different time points following the induction of experimental membranous nephropathy, and in control rats receiving normal sheep serum. At each time point, the number of adherent cells were quantitated after growing ex vivo for 24 hours under cell culture conditions. Figure 5 shows that cells were not detected in control rats injected with normal sheep serum. In PHN rats at days 1 and 3, the number of cells adherent to tissue culture plates was not significantly increased compared to control rats (Fig. 5). However, there was a marked and significant increase in cells when urine was collected from PHN rats at day 5 ($P < 0.001$ vs. control). The peak number of cells obtained from the urine from PHN rats that were adherent to cell culture plates was

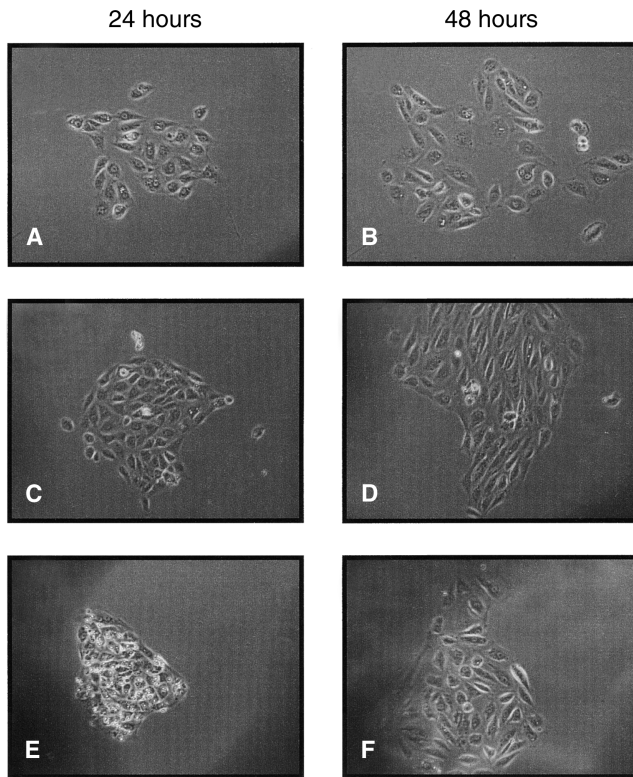


Fig. 4. Cells obtained from the urine of passive Heymann nephritis (PHN) rats grow under cell culture conditions. Cells obtained from the urine of PHN rats were resuspended in tissue culture medium, and grown under cell culture conditions. Cells obtained from the urine of three different PHN animals adhered to tissue culture plates at 24 hours ex vivo (A, C, and E). Cells have a typical cobblestone appearance. The same cells at 48 hours ex vivo (taken at the same magnification), and illustrate an increase in cell number, cell size, and mobility (B, D, and F).

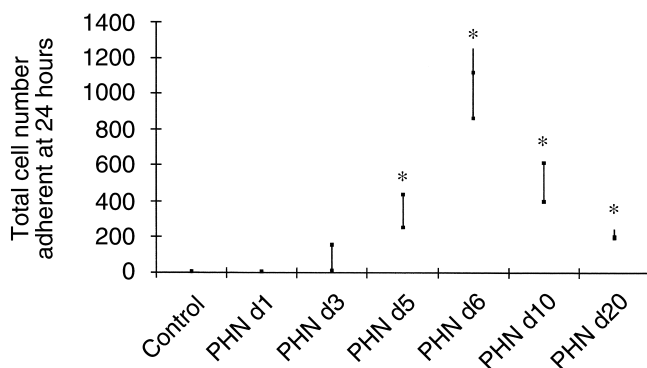


Fig. 5. Podocytes were detected in the urine of passive Heymann nephritis (PHN) rats at numerous time points. Urine was collected at different time points from PHN rats ($N = 6$ each time point), grown under cell culture conditions ex vivo, and total cell number was measured after 24 hours in cell culture on the adherent cells. Although cells were present at day 3, the increase was not statistically significant compared to control. There was a progressive and significant increase in cells obtained from the urine of PHN rats when grown ex vivo, with a peak in animals at day 6 of disease. Although the number declined at days 10 and 20 of disease, the number of cells remained statistically significantly compared to control rats injected with normal sheep serum. * $P < 0.01$ vs. control.

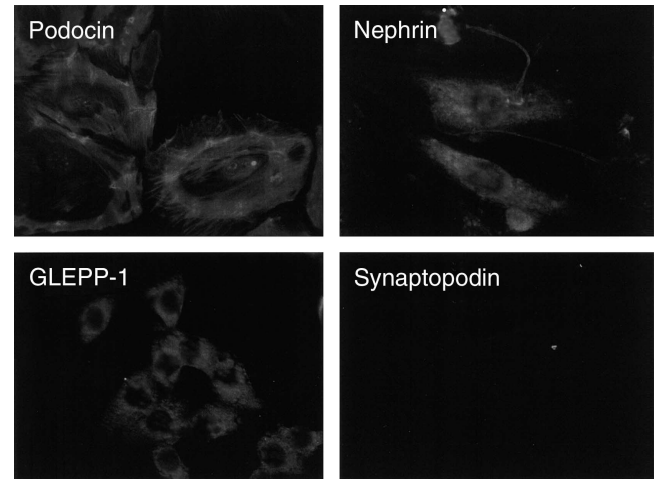


Fig. 6. Cells growing ex vivo stain positive for podocyte-specific markers. Cells obtained from the urine of passive Heymann nephritis (PHN) rats that adhered and grew on tissue culture plates ex vivo stained strongly positive for (A) podocin, (B) nephrin, GLEPP1 (C) and stained weakly for synaptopodin (D). These results show that cells are podocytes.

at day 6 ($P < 0.001$ vs. control). The number of cells that adhered to tissue culture plates remained significant when obtained from the urine of PHN rats at day 10 ($P < 0.001$ vs. control) and day 20 ($P < 0.001$ vs. control) (Fig. 5). These results show that viable cells were detected in the urine of rats from day 5 following the induction of experimental membranous nephropathy and this peaked on day 6 of disease and decreased at days 10 and 30.

Viable cells in the urine from PHN rats are podocytes

To identify which cell types were present in the urine and to determine which cell type(s) obtained from the urine were viable ex vivo, we performed immunostaining with podocyte, mesangial, endothelial, and tubular cell specific antibodies, and RT-PCR for immunostains that were positive.

Figure 6 shows that all adherent cells stained positively, and abundantly, for the podocyte-specific proteins podocin, nephrin, and GLEPP1. Cells also stained positively for synaptopodin, albeit more weakly than the other podocyte antigens shown in Figure 6. Rat mesangial cell cultures were used as negative controls (results not shown). Although immunostaining for WT-1 was detected in cells prior to plating them in culture, we were unable to detect positive immunostaining for WT-1 in podocytes growing ex vivo in culture.

We were unable to harvest sufficient protein from adherent cells to perform Western blot analysis. Accordingly, we performed RT-PCR on adherent cells for podocyte-specific antigens, and the results are shown in Figure 7. When total RNA was extracted from adherent cells

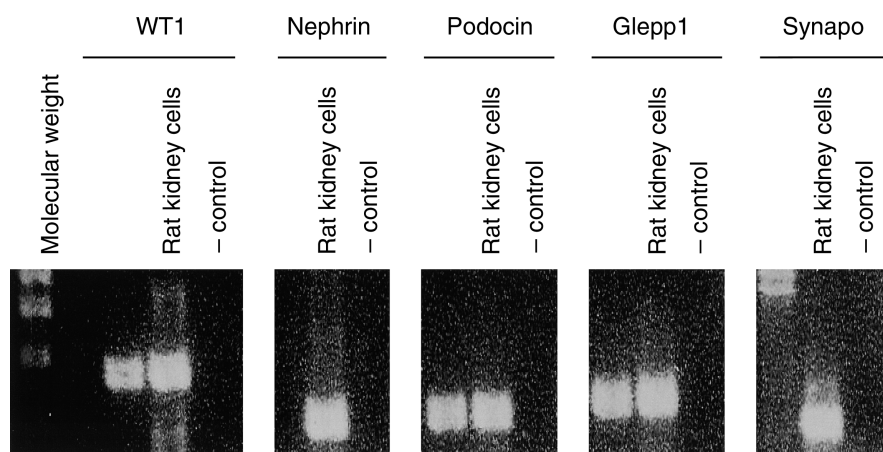


Fig. 7. mRNA expression for podocyte-specific antigens in cells growing ex vivo. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA extracted from cells growing ex vivo from PHN rats that had adhered to cell culture plates. mRNA expression was detected for WT-1, GLEPP1, and podocin, but not for nephrin and synaptopodin (synapo). The absence of transcripts were not false negatives, because the primers used detected nephrin and synaptopodin mRNA from normal rat kidney, used as a positive control. The negative control for each reaction comprised omitting the RT product and substituting this with water.

obtained from PHN rats, mRNA expression was detected for WT-1, GLEPP1, and podocin. In contrast, mRNA expression was not detected for nephrin and synaptopodin. These results were not false negatives, because mRNA expression for nephrin and synaptopodin were readily detected in RNA extracted from normal and PHN rat kidneys, used as positive controls.

Staining was also performed with antibodies to well-defined antigens on other renal cell types in control and PHN rats. Our results showed no detectable staining for Thy1 (mesangial cell antigen), RECA-1 (marker specific for glomerular endothelial cells), Tamm-Horsfall protein (marker of the thick ascending limb) and *Tetragonolobus purpureas* lectin (proximal tubular cell marker) in urine cultures from control and PHN rats (results not shown). The absence of immunostaining for these antigens was not due to the fixative or antibody used, because frozen rat kidney biopsies fixed similarly to cultured cells stained positive for each antibody (data not shown).

These results, summarized in Table 1, show that cells obtained from urine that adhered to cell culture plates expressed many podocyte-specific proteins, consistent with the hypothesis that viable podocytes detach in experimental membranous nephropathy.

Adherent cells have processes ex vivo

In addition to detecting the mRNA and protein expression of specific cell type markers, cell morphology was assessed serially by light and electron microscopy (Fig. 8). A number of observations were made. First, $28 \pm 5\%$ of urinary cells from PHN rats were polyploid. Second, beginning at day 3, morphologic changes included retraction of the cytoplasm from the cell periphery and thickening of the cell margins. This coincided with the appearance of cellular compartmentalization into a cell body, major processes, and minor processes. This was quite reminiscent of the typical in vivo podocyte architecture (Fig. 8F). Cell brush borders, characteristic of tubular

cells, were not detected on electron microscopy. Taken together with the immunostaining data described above, the electron microscopy suggests that the cells obtained from the urine of PHN rats are podocytes.

Detached podocytes proliferate ex vivo

We counted cell numbers every 12 hours in cells growing ex vivo from PHN rats and the results are shown in Figure 9 (the results from control rats are not reported because no adherent cells were detected at any of the time points studied). During the first 4 days of growing ex vivo, cell number increased every 12 hours under cell culture conditions, with the peak in cell number occurring at day 4. However, cell number decreased after day 4, and this coincided with an increase in the number of apoptotic cells (see below) (Fig. 9). Figure 8 provides further documentation of cell division in podocytes grown ex vivo, where cytokinesis was frequently observed. Shown is a quadronucleated cell undergoing cytokinesis resulting in two binucleated cells.

These results show that cells present in the urine of rats with experimental membranous nephropathy increased in number when grown under tissue culture conditions ex vivo, consistent with an increase in proliferation.

We determined if the decrease in cell number occurring from day 4 ex vivo was due to apoptosis, and the results are shown in Figure 9. The first increase in apoptosis was detected at 72 hours and persisted thereafter. These results show that cells derived from urine proliferate in culture, but the increase in cell number is limited by an increase in apoptosis.

Podocytes in urine from PHN rats stain positive for C5b-9

Podocyte injury in membranous nephropathy is due to C5b-9. Finally, in order to determine if detached podocytes expressed C5b-9, we performed immunostaining to C5b-9. All cells in the urine from PHN rats stained

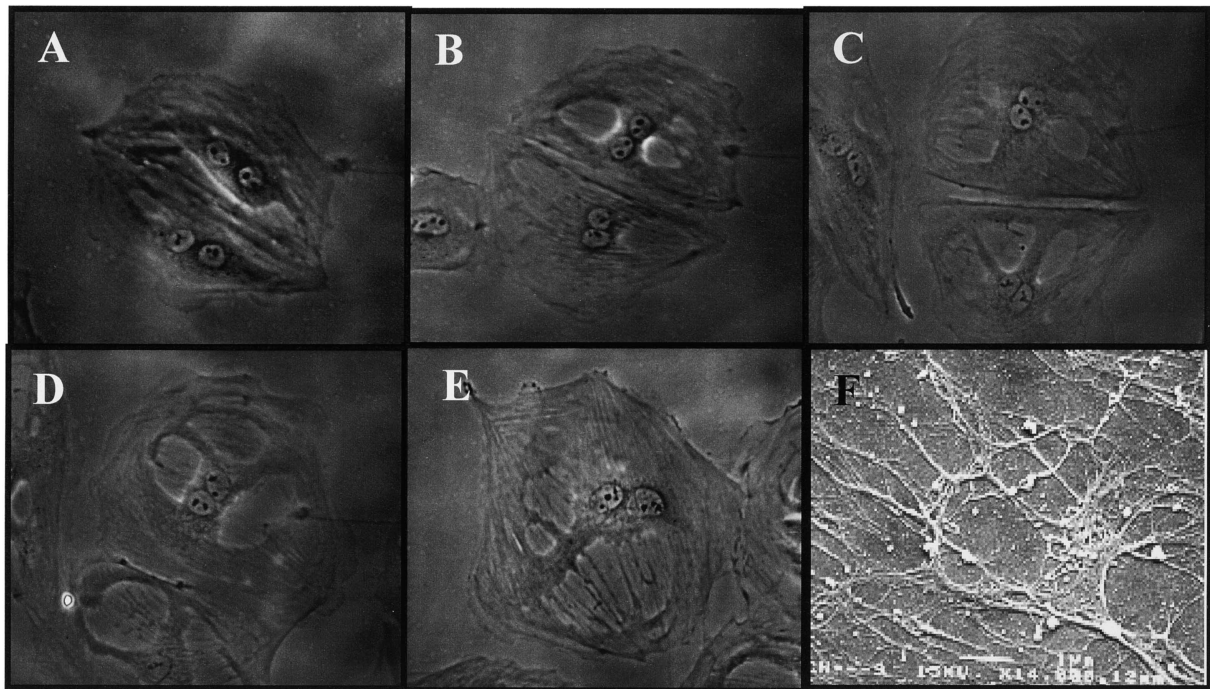


Fig. 8. Cell division in an individual multinucleated cell. (A to F) A temporal sequence of phase microscopy every 12 hours of a multinucleated cell dividing under cell culture conditions. Seen is a quadronucleated cell undergoing cytokinesis. Cytokinesis is followed by an increase in cell size and the development of a cell body with primary and secondary processes. Magnification of processes is shown by electron microscopy.

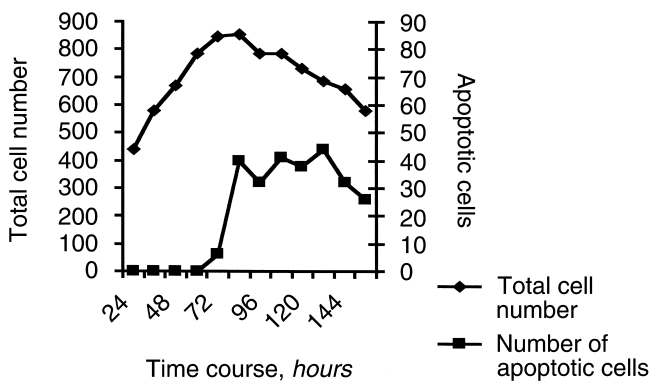


Fig. 9. Change of cell number and increase in apoptosis over time. Podocyte colonies were circled and individual cells counted all 12 hours. A peak of cell number is reached at around 72 hours. Decrease in cell number timely correlates with the appearance of apoptotic cells.

positive for C5b-9 (Fig. 2F). In contrast, cells from control rats were not detected.

DISCUSSION

Previous studies have demonstrated that the decrease in podocyte number is a critical determinant contributing to the development of glomerulosclerosis in diseases characterized by podocyte injury, such as membranous

nephropathy [3, 4, 9]. However, the mechanisms underlying podocyte loss remains to be fully elucidated. In this study, we show that podocytes are present in the urine of rats with experimental membranous nephropathy (PHN model) and that once they are detached from the glomerular basement membrane into the urinary space, they adhere to tissue culture plates *ex vivo*, indicating that they are viable.

Cells in the urine are not a novel observation. Studies three decades ago described the presence of cells in urine, and the authors interpreted these to be of tubular origin [24–28]. More recently, several groups have taken advantage of newer methodologies to document the presence of urinary podocytes in experimental and human glomerular disease [9, 17, 29,30]. However, the viability of these cells was not determined, and their proliferative capacity *ex vivo* has not been assessed. We began by showing for the first time that cells that express podocyte-specific antigens by immunostaining and RT-PCR are detected in the urine of rats with the PHN model of membranous nephropathy, but not in controls. The immunostaining and RT-PCR results were concordant. To determine if these cells were viable, cells were plated onto tissue culture plates and grown under cell culture conditions.

The major finding in the current study was that cells obtained from PHN rats adhered to cell culture plates,

and that they grew *ex vivo* under cell culture conditions. Moreover, using cell type specific antibodies, RT-PCR, and morphologic criteria, our results showed that the cells growing *ex vivo* were podocytes. These data show that podocytes in the urine of PHN rats were viable. We did observe some discrepancy between the immunostaining and RT-PCR results for WT-1, nephrin, and synaptopodin in adherent cells. First, despite abundant nephrin immunostaining in adherent cells, nephrin mRNA expression was not detected in these cells. Of note, nephrin mRNA was detected in cells obtained from PHN rat urine prior to being grown in culture (Fig. 3). Second, although synaptopodin immunostaining was barely detected in adherent cells, no mRNA expression was detected from adherent cells, despite the mRNA expression being detected when cells from the urine of PHN rats were studied prior to being committed to cell culture conditions. Finally, WT-1 immunostaining was positive in cells prior to the cell culture conditions, but was not detected once the cells adhered. In contrast, the mRNA expression that was detected prior to cell culture plating persisted in adherent cells. The reasons for these alterations in immunostaining are not known. One can speculate that we did not examine the correct time points or that the protein expression is altered *ex vivo*. Also, different regulatory mechanisms, including transcription and translation, might contribute.

In contrast to the presence of podocyte-specific antigens, we did not detect positive immunostaining for mesangial, endothelial, and tubular cell antigens. Although these cell types may have been present at low concentrations initially, the cell culture conditions selective for podocytes may favor podocyte growth over other cells. The final result was cell cultures uniformly staining positively for podocyte-specific proteins.

We asked if the loss of viable podocytes persisted throughout the course of disease. Our results also showed that loss of viable podocytes was present from day 5 of PHN, and that podocyte loss was ongoing up to day 20 of study in PHN rats. These results suggest that viable podocyte loss is ongoing. The consequence of this may be linked with podocyte number in PHN rats, because our data showed that there was a progressive decrease in podocyte number in PHN rats, which did not occur in controls. This decrease in glomerular podocyte number correlated with an increase in proteinuria at later time points. Thus, detachment of viable podocytes may be one explanation for the decrease in podocyte number. We did not examine the mechanisms underlying the detachment of viable podocytes. One may speculate a role for specific integrins, dystrophins, and other cellular events, which are currently under study.

A fundamental question is the proliferative capacity of podocytes. The prevailing notion is that podocytes do not typically proliferate *in vivo* [31, 32]. Indeed, studies

have shown that the limited proliferative potential of podocytes is a major contributor to the development of progressive glomerulosclerosis [6, 33]. A variety of potential mechanisms have been shown to limit podocyte proliferation *in vivo*, including an increase in specific cell cycle inhibitors. We and others have shown that podocytes increase cell cycle proteins required for DNA synthesis [7] and mitosis [10, 34]. Yet, there is a belief that podocytes do not divide (cytokinesis) *in vivo* in experimental and human membranous nephropathy. The results of the current study may shed new light into our understanding of podocyte proliferation. Our data showed that once viable podocytes from PHN rats are grown *ex vivo*, they increase in number during the first 60 hours of culture. These results suggest that when podocytes are no longer adherent to the glomerular basement membrane *in vivo*, they have the capacity to proliferate. This raises the possibility that podocyte attachment to the underlying glomerular basement membrane may be a limiting factor in this cell's proliferative capacity. Studies are needed to elucidate the mechanisms of podocyte proliferation *ex vivo*.

Despite proliferation *ex vivo*, cell number decreased with time due to increased apoptosis. It is unclear if the switch from a predominantly proliferative phenotype to an apoptotic one is due to the cell culture conditions, the cells limited proliferative capacity (such as occurs *in vivo*), or the loss of survival factors. Finally, because a large number of podocytes were bi- and multinucleated *in vitro* (similar to that described *in vivo* in PHN rats) [35], another possibility is that only polyploid cells are able to complete cytokinesis *ex vivo*, and thus proliferate, and that polyploid cells are the phenotype primarily lost in the urine.

What are the clinical implications of our study? One possibility is that detecting viable podocytes in urine may assist with the diagnosis of diseases of the podocyte. Second, we showed that podocytes from PHN rats stained positive for C5b-9. This finding is specific to membranous nephropathy and might assist with a prebiopsy diagnosis. One other potential application is the detection of gene product defects, such as nephrin or podocin, in patients with congenital diseases.

CONCLUSION

This study shows that podocytes detach in experimental membranous nephropathy and are viable. They also have a limited proliferative capacity *ex vivo*, before undergoing apoptosis. Further studies are needed to delineate the mechanisms underlying podocyte detachment and to determine if attachment to the glomerular basement membrane limits proliferation.

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REFERENCES

- KERJASCHKI D: Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* 108:1583–1587, 2001
- MUNDEL P, SHANKLAND SJ: Podocyte biology and response to injury. *J Am Soc Nephrol* 13:3005–3015, 2002
- PAGTALUNAN ME, MILLER PL, JUMPING-EAGLE S, et al: Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99:342–348, 1997
- LEMLEY KV, LAFAYETTE RA, SAFAI M, et al: Podocytopenia and disease severity in IgA nephropathy. *Kidney Int* 61:1475–1485, 2002
- STEFFES MW, SCHMIDT D, MCCRERY R, BASGEN JM: Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* 59:2104–2113, 2001
- KRIZ W: Progressive renal failure-inability of podocytes to replicate and the consequences for development of glomerulosclerosis. *Nephrol Dial Transplant* 11:1738–1742, 1996
- SHANKLAND SJ, FLOEGE J, THOMAS SE, et al: Cyclin kinase inhibitors are increased during experimental membranous nephropathy: Potential role in limiting glomerular epithelial cell proliferation in vivo. *Kidney Int* 52:404–413, 1997
- SCHIFFER M, BITZER M, ROBERTS IS, et al: Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* 108:807–816, 2001
- KIM YH, GOYAL M, KURNIT D, et al: Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int* 60:957–968, 2001
- PIPPIN JW, DURVASULA R, PETERMANN A, et al: DNA damage is a novel response to sublytic complement C5b-9-induced injury in podocytes. *J Clin Invest* 111:877–885, 2003
- KIHARA I, TSUCHIDA S, YAOITA E, et al: Podocyte detachment and epithelial cell reaction in focal segmental glomerulosclerosis with cellular variants. *Kidney Int* 52 (Suppl 63):S171–S176, 1997
- NAKAMURA T, USHIYAMA C, SUZUKI S, et al: Urinary podocytes for the assessment of disease activity in lupus nephritis. *Am J Med Sci* 320:112–116, 2000
- NAKAMURA T, USHIYAMA C, SUZUKI S, et al: Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol Dial Transplant* 15:1379–1383, 2000
- NAKAMURA T, USHIYAMA C, SUZUKI S, et al: The urinary podocyte as a marker for the differential diagnosis of idiopathic focal glomerulosclerosis and minimal-change nephrotic syndrome. *Am J Nephrol* 20:175–179, 2000
- HARA M, YANAGIHARA T, TAKADA T, et al: Urinary excretion of podocytes reflects disease activity in children with glomerulonephritis. *Am J Nephrol* 18:35–41, 1998
- HARA M, YANAGIHARA T, KIHARA I: Urinary podocytes in primary focal segmental glomerulosclerosis. *Nephron* 89:342–347, 2001
- VOGELMANN SU, NELSON WJ, MYERS BD, LEMLEY KV: Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol* 285:F40–F48, 2003
- WHITE KE, BLOUS RW, MARSHALL SM, et al: Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* 51:3083–3089, 2002
- BRADLEY GM, BENSON ES: *Examination of the Urine*, 15th ed., Philadelphia, WB Saunders, 1974
- HIROMURA K, PIPPIN JW, BLONSKI MJ, et al: The subcellular localization of cyclin dependent kinase 2 determines the fate of mesangial cells: Role in apoptosis and proliferation. *Oncogene* 21:1750–1758, 2002
- HOYER JR, FOGO AB, TERRELL CH, DELANEY MM: Immunomorphometric studies of proteinuria in individual deep and superficial nephrons of rats. *Lab Invest* 80:1691–1700, 2000
- QUAGGIN SE, SCHWARTZ L, CUI S, et al: The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. *Development* 126:5771–5783, 1999
- KIM Y-G, PIPPIN JW, JOHNSON RJ, et al: The cyclin kinase inhibitor p21Cip1/WAF1 limits visceral glomerular epithelial cell proliferation in experimental glomerulonephritis. *Kidney Int* 55:2349–2361, 1999
- SUTHERLAND GR, BAIN AD: Cultures of cells from the urine of newborn children. *Nature* 239:231, 1972
- LINDNER D: Culture of cells from the urine and bladder washings of adults. *Somatic Cell Genet* 2:281–283, 1976
- FELIX JS, LITTLEFIELD JW: Urinary tract epithelial cells cultured from human urine. *Int Rev Cytol* 60(Suppl):11–23, 1979
- FELIX JS, LITTLEFIELD JW: Human epithelial cells cultured from urine: Growth properties and keratin staining. *In Vitro* 16:866–874, 1980
- SINS DA, DETRISAC CJ, MAYFIELD RK, COLWELL JA: In vitro culture of cells exfoliated in the urine by patients with diabetes mellitus. *J Clin Invest* 71:170–173, 1983
- EBIHARA I, NAKAMURA T, USHIYAMA C, et al: Urinary podocytes in patients with chronic renal failure. *Nephron* 85:187, 2000
- HARA M, YANAGIHARA T, KIHARA I: Urinary podocytes in primary focal segmental glomerulosclerosis. *Nephron* 89:342–347, 2001
- NAGATA M, YAMAGUCHI Y, ITO K: Loss of mitotic activity and the expression of vimentin in glomerular epithelial cells of developing human kidneys. *Anat Embryol* 187:275–279, 1993
- NAGATA M, NAKAYAMA K-I, TERADA Y, et al: Cell cycle regulation and differentiation in the human podocyte lineage. *Am J Pathol* 153:1511–1520, 1998
- NAGATA M, TOMARI S, KANEMOTO K, et al: Podocytes, parietal cells, and glomerular pathology: The role of cell cycle proteins. *Pediatr Nephrol* 18:3–8, 2003
- PETERMANN AT, PIPPIN J, HIROMURA K, et al: Mitotic cell cycle proteins increase in podocytes despite lack of proliferation. *Kidney Int* 63:113–122, 2003
- KRIZ W, HAHNEL B, ROSENER S, ELGER M: Long-term treatment of rats with FGF-2 results in focal segmental glomerulosclerosis. *Kidney Int* 48:1435–1450, 1995